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## Review

# Chloride channelopathies

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## ABSTRACT

Channelopathies, defined as diseases that are caused by mutations in genes encoding ion channels, are associated with a wide variety of symptoms. Impaired chloride transport can cause diseases as diverse as cystic fibrosis, myotonia, epilepsy, hyperekplexia, lysosomal storage disease, deafness, renal salt loss, kidney stones and osteopetrosis. These disorders are caused by mutations in genes belonging to non-related gene families, i.e. CLC chloride channels and transporters, ABC transporters, and GABA- and glycine receptors. Diseases due to mutations in TMEM16E and bestrophin 1 might be due to a loss of Ca<sup>2+</sup>-activated Cl<sup>-</sup> channels, although this remains to be shown.

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## 1. Introduction

Although the best known function of ion channels is probably that in electrical excitability of nerve and muscle, they play much broader roles. At the cellular level, they are for instance important for the regulation of cell volume, cytoplasmic and vesicular pH, and for the regulation of cytoplasmic Ca<sup>2+</sup>-concentration that is crucial in intracellular signaling. At the level of the organism, ion channels are involved for instance in transepithelial transport, secretion of hormones, and bone metabolism. It therefore does not come as a surprise that mutations in genes encoding ion channels lead to a plethora of very different inherited diseases that are known as 'channelopathies'.

Ion channels can be studied in great detail by biophysical techniques which include the analysis of single channel molecules by patch-clamping. Electrophysiological analyses of ion channels engineered to carry human disease mutations have yielded important insights into the pathological mechanisms of channelopathies – often much more than is possible with other human inherited diseases. However, one should add a word of caution: the subcellular localization and biophysical properties of ion channels can be influenced by the cell in which they are expressed, and by various experimental parameters. Therefore, the properties of heterologously expressed ion channel mutants do not always reflect faithfully the

situation in the patient. Given that detailed understanding of the effect mutations have on ion channel properties is mostly not sufficient to understand the pathology at the level of the organism, knock-out mice deleted for the respective ion channel gene have been generated, or even knock-in mice carrying the equivalent of a human disease mutation. Explorative knock-outs often revealed pathologies akin to known human diseases and sometimes led to the identification of a human disease gene.

Ion channels, which are defined as integral membrane proteins displaying a 'pore' that allows the passive movement of ions at high transport rates, constitute just one class of ion transport proteins. Other ion transporters can couple the movement of one ion to another one – either as cotransport into the same direction (symporter), or in an exchange process (antiporter). Whereas these transporters are also 'passive', so-called 'pumps' use the energy derived e.g. from ATP hydrolysis to drive ion transport against an electrochemical gradient. In channelopathies, these other transport molecules must be taken into account to understand the pathological consequences of ion channel mutations. In some cases, a single gene family can encode ion channels as well as exchangers. This is e.g. the case with the CLC family that is now known to encode Cl<sup>-</sup> channels and Cl<sup>-</sup>/H<sup>+</sup>-exchangers. In this case we opted for also describing diseases caused by mutations in CLC exchangers, although they do not conform strictly to the definition of 'channelopathy'.

In general, anion channels have found less interest than cation channels, probably because they were rather regarded as a nuisance when studying action potentials of nerve cells. On the other hand, ligand-gated anion channels (Gamma Amino Butyric Acid [GABA]- and

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glycine-receptors) are crucial for neuronal inhibition. Many chloride channelopathies were discovered during the last 15 years or so. These cause symptoms as diverse as epilepsy, startle disease, deafness, blindness, lysosomal storage and neurodegeneration, osteopetrosis, lung infections and fibrosis, male infertility, renal salt loss, and kidney stones, clearly indicating the crucial importance of anion transport in many tissues. Rather than structuring our review along disease phenotypes, we opted to discuss channelopathies in the framework of the corresponding gene families.

## 2. CLC chloride channels and transporters

Several human inherited diseases are caused by mutations in CLC chloride channels or transporters. First identified by cloning the voltage-gated chloride channel CLC-0 from the electric fish *Torpedo marmorata* [1], the CLC gene family is found in all phyla, from bacteria to man. CLC proteins function as chloride channels or  $\text{Cl}^-/\text{H}^+$  exchangers. CLC channels and exchangers operate as dimers with two largely independent permeation pathways. There are nine different CLC genes in mammals (for a review, see [2]). They encode either plasma membrane chloride channels, or transporters that are mainly localized to intracellular compartments like endosomes, lysosomes or synaptic vesicles. It seems very likely that all of these mainly intracellular CLC proteins are  $\text{Cl}^-/\text{H}^+$ -exchangers rather than chloride channels [3].

Human mutations in *CLCN* genes (genes are called *CLCN1*, *CLCN2* etc. and encode the proteins CLC-1, CLC-2 etc) cause genetic diseases with symptoms as diverse as myotonia, kidney stones, renal salt loss, deafness, osteopetrosis and lysosomal storage disease. These diseases are reviewed below. In addition, KO mouse models have revealed phenotypes for which no corresponding human disease has been identified so far: the loss of the plasma membrane  $\text{Cl}^-$  channel CLC-2 causes male infertility, retinal degeneration and leukodystrophy [4,5] The selective loss of the plasma membrane  $\text{Cl}^-$  channel CLC-K1 leads to a diabetes insipidus-like syndrome [6]. Disruption of the endosomal/synaptic vesicle CLC-3 entails a severe neurodegeneration [7] and a secretory defect in neurosecretory cells [8], whereas mice lacking the late endosomal CLC-6 display a distinct form of neuronal lysosomal storage disease [9]. These phenotypes illustrate the diverse and important roles of chloride transport over cellular membranes and have been reviewed in depth elsewhere [2,10].

### 2.1. CLC-1 and myotonia

Myotonia, a symptom of several skeletal muscle diseases, is characterized by 'muscle stiffness' owed to an electrical hyperexcitability of the muscle membrane that is apparent in the form of 'myotonic runs' or 'discharges'. After voluntary contraction, muscles do not relax properly. Myotonia is associated with dystrophic and other features in myotonic dystrophy, can be precipitated by cold temperatures as in paramyotonia congenita, may be aggravated by potassium, or is found in hyperkalemic paralysis with myotonia. With the exception of myotonic dystrophy, all these genetic syndromes are caused directly by mutations in ion channel genes [11]. Pure myotonia congenita, in both its recessive (Becker type) or in its less severe dominant form (Thomsen's disease) is caused by mutations in the *CLCN1* gene that encodes the skeletal muscle chloride channel CLC-1 [12–15].

CLC-1 is almost exclusively expressed in skeletal muscle [16]. CLC-1 is a voltage-gated chloride channel with a low single channel conductance of about 1 pS. It displays the typical double-barrel appearance of CLC channels with two largely independent pores [17,18]. CLC-1 is already open under resting membrane potential, but can be further activated by depolarization [16]. CLC-1 currents observed in heterologous expression systems [16,19] closely resemble  $\text{Cl}^-$  currents in native skeletal muscle fibers [20]. The modulation of

CLC-1 activity by ATP and the redox potential may play a role in muscle fatigability [21]. The surprisingly high chloride conductance (~80% of total conductance at rest) that is conferred to skeletal muscle by CLC-1 is important to repolarize action potentials and to stabilize its resting voltage. Why is there such a high resting chloride conductance in skeletal muscle, as opposed to the high resting potassium conductance in most other cells? This finding may be related to the repolarization of action potentials in transverse tubules (t-tubules), fine tubular extensions of the plasma membrane that carry electrical excitation deep into the muscle fiber to trigger calcium release from intracellular stores close to the contraction machinery. If repolarization of action potentials would be carried by an efflux of potassium, there would be a substantial increase of  $[\text{K}^+]_o$  in these tubules, with the potential to depolarize this membrane [22]. As extracellular chloride concentration is more than 20-fold higher than that of potassium, its relative increase and hence its effect on membrane potential is much less pronounced. However, although skeletal muscle chloride conductance was severely decreased by tubular disruption with glycerol [23], immunohistochemistry failed to detect CLC-1 in t-tubules, rather finding it in the (outer) sarcolemma instead [24,25]. This problem remains to be resolved.

Following reports that skeletal muscle chloride conductance was reduced in some forms of myotonia of animal models and human patients [26–28], CLC-1 was first shown to be mutated in a spontaneous myotonic mouse mutant [12], then in humans [14] and later in goats [29] and dogs [30]. In humans, mutations have been found in both the recessive (Becker) and dominant (Thomsen's disease) forms of disease [14,15,31]. Even the *CLCN1* mutation of Dr. Thomsen, who first described the disease in 1876 and who was affected himself, was identified and characterized [13].

Mutations that lead to an early truncation of the CLC-1 protein and thus to a total loss of  $\text{Cl}^-$  channel activity cause recessive (Becker-type) myotonia. This suggests that a reduction of muscle  $\text{Cl}^-$  conductance down to 50% is still compatible with normal muscle excitability. Recessive myotonia can also be caused by many different missense mutations in CLC-1 [31]. The mechanisms underlying a loss of channel function include impaired transport to the plasma membrane, altered voltage dependence, and reduced single channel conductance [32,33].

Dominant myotonia can be explained by mutant subunits (encoded on one allele) which can inhibit the function of heteromers that are formed with WT subunits encoded by the other allele [13]. As CLC channels are dimers [34–36], one expects that in heterozygous patients, 25% fully functional WT/WT channels will remain. Indeed, myotonia is clinically less severe in dominant compared to recessive forms. The double-barreled architecture of CLC channels, where each subunit contains a pore of its own, leave much less room for dominant-negative effects compared to  $\text{K}^+$ -channels where four subunits form a common, central pore. Many dominant CLC-1 mutations lead to a large reduction of chloride conductance at physiological voltages by shifting the voltage-dependence of the channel to positive voltages [37]. As expected, such mutations impinge on the common gate that acts on both pores of the dimer [18]. When crystal structures from bacterial CLCs became available [36], it was realized that in many dominant mutations there are amino acid exchanges close to the dimer interface [38]. Some mutations can be found in pedigrees with either apparently recessive or dominant inheritance. These mutations lack full penetrance in a heterozygous state. They may result in this 'intermediate' phenotype by only moderately shifting the voltage-dependence of WT/mutant heteromers [39].

Nucleotide repeats in untranslated regions of two different genes, encoding the protein kinase DMPK or the zinc finger protein-9 (ZFN9), underlie myotonic dystrophy. Aberrant transcripts of either gene are retained in the nucleus and affect pre-mRNA splicing, probably by sequestration of RNA-binding proteins. Myotonia, a cardinal symptom

of that multisystem disorder, has been attributed to aberrant splicing of CIC-1.

This leads to a drastic decrease in both CIC-1 protein levels [40,41] and currents [20]. In mouse models of myotonic dystrophy, the aberrant splicing of CIC-1 could be suppressed by an antisense morpholino oligonucleotide, increasing CIC-1 current density and eliminating myotonic discharges [42].

## 2.2. CIC-2: a role in epilepsy?

The CIC-2 chloride channel is broadly expressed across many tissues [43]. In the brain, it is found in neurons and glia, including astrocytes and oligodendrocytes [5,44]. CIC-2 is activated by hyperpolarization [43], cell swelling [45], and moderately acidic extracellular pH [46]. CIC-2 currents are augmented by an increase in intracellular chloride concentration ( $[Cl^-]_i$ ) [47–49]. CIC-2 is a typical 'double-barreled' channel with two  $\sim 3$  pS pores [50].

In view of its expression in a broad range of tissues, many functions have been assigned to CIC-2, often without proper experimental support. So, the purported roles of CIC-2 in gastric acid secretion [51] or in lung development [52] were not supported by the phenotype of CIC-2 KO mice [4]. As CIC-2 is expressed in neurons, it was thought to contribute to the establishment of low intraneuronal chloride concentration, a prerequisite for the inhibitory response to GABA and glycine [53,54]. Because GABA<sub>A</sub>- and glycine-receptors are ligand-gated chloride channels, the electrochemical chloride gradient across the plasma membrane determines whether GABA or glycine depolarize (with high  $[Cl^-]_i$ ) or hyperpolarize (with low  $[Cl^-]_i$ ) the postsynaptic membrane. Indeed, transfection of CIC-2 into dorsal root ganglia neurons, which in contrast to most other neurons maintain a depolarizing GABA-response in adult life, changed their GABA response towards inhibition [54]. It is obvious that the expression of a large chloride conductance should equilibrate  $[Cl^-]_i$  close to its electrochemical potential. Opening additional GABA-receptor chloride channels will then clamp the membrane voltage to its resting value, resulting in neuronal inhibition. On the other hand, it is obvious that CIC-2 cannot decrease  $[Cl^-]_i$  below its equilibrium value. Such a low  $[Cl^-]_i$ , as observed in the vast majority of adult (but not early) neurons, is the basis for GABA- and glycine-mediated hyperpolarization. In fact it is now clear that the KCl-cotransporter KCC2 plays the major role in establishing low intraneuronal  $[Cl^-]$  in adult neurons [55,56].

When Sander et al. identified a susceptibility locus for idiopathic epilepsy on human chromosome 3q26 [57], a region where CIC-2 is encoded, the *CLCN2* gene was considered as a candidate gene for human epilepsy. Indeed, if CIC-2 was important for inhibitory GABA response, its disruption might lead to an increased neuronal excitability. Haug et al. [58] identified three CIC-2 sequence abnormalities in three families with different clinical forms of idiopathic epilepsy: one premature stop-codon, one intronic deletion of 11 base pairs in the vicinity of an exon–intron boundary, and one missense mutation (G715E) that affects a non-conserved residue in the cytoplasmic tail. The intronic variant reportedly increased the proportion of a non-functional splice variant that was also found in control patients, thereby predicting a partial loss of function [58]. The truncated CIC-2 protein was described to exert a dominant negative effect on co-expressed WT channels. However, this effect could not be found in other laboratories [5,59]. The effect of the intronic deletion on CIC-2 splicing could not be confirmed, either [59]. The G715E mutation was reported to cause a gain of channel function by shifting the voltage-dependence of CIC-2 to more positive voltages [58]. This led to the surprising conclusion that both loss and gain of CIC-2 function may result in epilepsy. However, this effect of the G715E mutation could not be reproduced either [59]. So far no convincing *CLCN2* mutations have been identified in epileptic patients following the original report [60–63]. *CLCN2* sequence abnormalities identified in epileptic patients [62] were later also found in persons lacking

epilepsy [5,63]. These sequence variants did not change CIC-2 currents when tested in heterologous expression, either [5]. Thus, a heterozygous, premature stop in a small family [58] is the one remaining mutation that unambiguously causes a loss of CIC-2 function. As the truncated protein lacks dominant negative effects [5,59], it would have to cause epilepsy by haploinsufficiency. However, even the complete loss of CIC-2 in mice does not result in epilepsy or reduced seizure thresholds [4,5]. In conclusion, the evidence that mutations in *CLCN2* underlie human epilepsy is not yet convincing.

Disruption of CIC-2 in mice gave unexpected results that do not support a role of this channel in epilepsy. These mice are blind and display male infertility [4]. The retina of *Clcn2*<sup>−/−</sup> mice degenerates early after birth. There is a severe degeneration of the testes that leads to a total loss of spermatocytes and spermatogonia [4]. Both phenotypes were tentatively attributed to a defect in transepithelial transport across retinal pigment epithelial cells and Sertoli cells, respectively, which eventually lead to the death of photoreceptors and germ cells due to a changed extracellular environment [4]. CIC-2 KO mice also display leukodystrophy that is associated with the appearance of vacuoles in central myelin sheaths [5]. As their leukoencephalopathy does not lead to significant neuronal cell death, the neurological symptoms of these mice (with the exception of blindness) are mild and include a decreased speed of nerve conduction in the CNS [5]. In analogy to other forms of leukodystrophy that are caused by mutations in the Kir4.1 K<sup>+</sup>-channel or in certain gap junction proteins, it was hypothesized that CIC-2 is important for extracellular ion homeostasis in the narrow clefts between nerve cells and glia [5]. This would fit with the expression of CIC-2 in astrocytic endfeet that contact small brain capillaries [5,44] and with the role that was postulated for CIC-2 in the testes and retina [4]. While further genes for human leukodystrophy remain to be discovered, no *CLCN2* mutations were found in a large collection of patients with leukodystrophy [5].

CIC-2 has previously been speculated to provide an alternative pathway for chloride secretion in epithelia of patients with cystic fibrosis [64]. However, mice homozygous for the CFTR  $\Delta F508$  mutation and null for CIC-2 did not display more severe, but rather slightly milder intestinal problems (the CF phenotype observed in mice) [65]. This was attributed to a basolateral expression of CIC-2, where it does not act synergistically with the apical CFTR channel. Although there are divergent results [66], CIC-2 has been convincingly identified in basolateral membranes of intestinal epithelial cells [67–69] (Zdebik and Jentsch, unpublished) and of salivary duct cells [70]. The apical expression of CIC-2 reported for airway epithelial cells [69] remains to be confirmed using knock-out controls.

## 2.3. CIC-K/barttin chloride channels: salt loss and deafness

Human Bartter syndromes constitute a group of rare, genetically heterogeneous autosomal nephropathies that are associated with renal salt wasting, elevated renin and aldosterone levels, and typically hypokalemic metabolic alkalosis (for reviews of different forms of Bartter syndrome, see [71,72]). Their common pathophysiological mechanism is an impairment of NaCl reabsorption in the thick ascending limb of Henle's loop (TAL). Bartter syndromes type I to IV are caused by mutations in ion channels and transporters that are expressed in renal epithelia, in particular the TAL. Certain activating mutations in the extracellular Ca<sup>++</sup>-sensing receptor CaSR can also cause an autosomal dominant Bartter-like syndrome that was called Bartter V [73,74]. The activation of this G-protein coupled receptor inhibits transepithelial salt transport in the TAL. Mutations in *CLCNKB* encoding the Cl<sup>−</sup> channel  $\alpha$ -subunit CIC-Kb underlie Bartter syndrome type III. Bartter IV, which combines even more severe renal symptoms with congenital deafness, is either caused by mutations in the Cl<sup>−</sup> channel  $\beta$ -subunit barttin (encoded by the *BSND* gene), or by loss of function mutations in both CIC-Ka and CIC-Kb.

The tissue distribution of CIC-Ka and CIC-Kb (CIC-K1 and -K2 in rodents) is restricted to the kidney (hence the K in their name) and the inner ear. The genes encoding these highly related isoforms (~90% identity at the protein level) [75–77] map to human chromosome 1p36 [78] and are separated by only a few kilobases [79]. This observation explains that even rather small chromosomal deletions can entail a functional loss of both isoforms [80,81] or to a fusion protein under the control of the CIC-Ka promoter [79]. Both CIC-Ka and CIC-Kb need barttin, a small ~40 kDa protein that spans the membrane twice [82], for their transport to the plasma membrane [83]. In addition to this 'chaperone' effect, barttin influences the biophysical properties of CIC-K currents [84,85]. In contrast to other CLC channels, CIC-K/barttin chloride channels are nearly devoid of voltage-dependent gating [83], an observation that fits well to the fact that the 'gating glutamate' that is present in almost all CLC proteins is replaced by valine or isoleucine in CIC-K proteins. The resulting ohmic current is well suited for the constitutive, transepithelial transport that is carried out by these channels both in the kidney and the inner ear.

Whereas both CIC-K isoforms are apparently co-expressed in inner ear epithelia (marginal cells of the stria vascularis in the cochlea and dark cells of the vestibular organ) [83,86], they show differential distribution along the nephron. CIC-Ka (-K1) is mainly expressed in the thin limb of Henle's loop [6,87], where it probably mediates the high chloride conductance of that segment. This conductance contributes to the establishment of a high osmolarity of the kidney medulla, which in turn is needed for urinary concentration. Indeed, CIC-K1 KO mice are impaired in their ability to concentrate urine after water deprivation [6]. This phenotype resembles mild diabetes insipidus in humans, but no isolated mutation in *CLCNKA* has been reported so far.

CIC-Kb, in contrast, is prominently expressed in the thick ascending limb of Henle's loop, a main site of NaCl reabsorption. In addition, it is found in the distal tubule and in intercalated cells of the collecting duct [88,89]. In all these cells, it is strictly localized in their basolateral membrane. Whereas the role of CIC-Kb/barttin in distal nephron segments is incompletely understood, the observation that loss-of-function mutations in *CLCNKB* lead to Bartter syndrome type III in humans [79] strongly suggests that CIC-Kb/barttin is crucial for NaCl reabsorption in the thick ascending limb. It provides the basolateral exit pathway for chloride that is taken up across the apical membrane by the NaK2Cl cotransporter NKCC2. This transport model is strongly supported by human mutations in *NKCC2* that lead to Bartter syndrome type I [90], and in *KCNJ1* in Bartter syndrome II [91]. The latter gene encodes the ROMK (Kir1.1) K<sup>+</sup>-channel that is necessary for the apical recycling of potassium that is cotransported by NKCC2. The severe impairment of NaCl reabsorption in the thick limb leads to a secondary stimulation of Na<sup>+</sup>-reabsorption through ENaC in the distal nephron. The resulting depolarization of ENaC-expressing principal cells is thought to stimulate K<sup>+</sup> secretion through ROMK channels that are co-expressed with ENaC in their apical membrane. This explains the hypokalemia observed in Bartter I and III, and its absence in Bartter II patients who lack functional ROMK channels.

Mutations in *BSND*, the gene encoding barttin, a  $\beta$ -subunit common to both CIC-K isoforms [83], lead to Bartter IV [82]. This syndrome combines renal salt and fluid loss with congenital deafness. Because the loss of barttin will disrupt both CIC-Ka/barttin and CIC-Kb/barttin, renal symptoms are more severe in Bartter IV than in Bartter III and often lead to renal failure. Inner ear epithelia express both CIC-K isoforms [83]. This redundancy explains that the loss of solely CIC-Kb, as in Bartter III, does not lead to deafness. Only when both CIC-Ka/barttin and CIC-Kb/barttin are lost, as with mutational inactivation of barttin [82] or with the rare patients who have lost both CIC-K isoforms due to chromosomal deletions [80,81], congenital deafness ensues. The mechanism of deafness in Bartter IV has recently

been elucidated with a mouse model in which barttin was specifically deleted in the inner ear, but not in the kidney [92]. Like patients, these mice were congenitally deaf. They displayed a stable hearing loss of about 60 dB. This hearing loss could be attributed to a severe breakdown of the endocochlear potential (EP) that normally maintains the fluid space of the scala media roughly 100 mV more positive than normal extracellular space. Rather unexpectedly, however, the endocochlear potassium concentration ([K<sup>+</sup>]) was normal [92]. Both properties, i.e. high EP and endolymphatic [K<sup>+</sup>], are of utmost importance for hearing. In sensory hair cells, the depolarizing current through apical mechanosensitive channels is carried by K<sup>+</sup>, and not by Na<sup>+</sup> as in other cells like neurons or muscle. This may be rationalized in terms of energy requirements of the organ of Corti: if these cells were depolarized by an influx of sodium, Na<sup>+</sup> would need to be extruded continuously by the energy-consuming Na,K-ATPase. The necessary supply of metabolic energy would require the organ of Corti to be vascularized, which would interfere with the micromechanics of hearing. The creation of two different compartments in contact with hair cells – apical endolymph with high [K<sup>+</sup>] and potential, and the endolymph that exposes the basolateral membrane of hair cells to normal [K<sup>+</sup>] of 5 mM and 0 mV – allows for a passive influx and efflux of K<sup>+</sup>. Nature has put the battery driving this system into the lateral wall of the scala media – the epithelium of the stria vascularis that generates both the high [K<sup>+</sup>] and the EP. This epithelium consists of three layers: the marginal cells facing the endolymph are closely interdigitated with intermediate cells, which are in turn connected by gap junctions with underlying basal cells. Marginal cells secrete K<sup>+</sup> through their apical KCNQ1/KCNE1 K<sup>+</sup>-channels. Potassium is taken up across their basolateral membrane by the combined activity of the Na,K-ATPase and the NaK2Cl cotransporter NKCC1. The latter transporter also accumulates chloride, which needs to be recycled over the basolateral membrane by CIC-Ka/barttin and CIC-Kb/barttin Cl<sup>-</sup> channels. A disruption of this recycling pathway is therefore expected to block strial K<sup>+</sup>-secretion, similar to what has been found with loss-of-function mutations in KCNQ1, KCNE1, or NKCC1. The normal endolymphatic [K<sup>+</sup>] and the normal position of Reissner's membrane that is an indicator of strial fluid secretion, however, suggests that strial K<sup>+</sup>-secretion is not totally abolished. This suggests that marginal cells express another pathway for Cl<sup>-</sup> exit, e.g. a swelling- or Ca<sup>++</sup>-activated Cl<sup>-</sup> channel. As another mouse model that additionally led to partial deletion of barttin in the kidney showed a collapse of Reissner's membrane, it was inferred that strial K<sup>+</sup>-secretion is indeed reduced, but not abolished [92]. A more severe reduction secondary to renal salt and fluid loss tips the balance to a point where K<sup>+</sup>-secretion no longer suffices to maintain the endolymphatic space.

The endocochlear potential is largely generated by a K<sup>+</sup>-diffusion potential across apical Kir4.1 K<sup>+</sup> channels of intermediate cells [93,94]. The voltage across that membrane reaches some 100 mV because [K<sup>+</sup>] in the intrastrial space between marginal and intermediate cells is particularly low owed to the avid uptake of K<sup>+</sup> by marginal cells. If this uptake is compromised as in the barttin KO, intrastrial [K<sup>+</sup>] will rise, resulting in a drastic decrease of the EP. Further components contributing to the decrease of EP are probably the lack of a depolarizing basolateral chloride current carried by CIC-K/barttin, and a decrease of [K<sup>+</sup>]<sub>i</sub> in marginal cells [92]. Because sensory hair cells (like almost all other cells) have an inside-negative membrane potential, the combination of normal endolymphatic [K<sup>+</sup>] with a severely reduced EP should reduce, but not abolish the transduction current. Nonetheless, this reduction was sufficient to abolish otoacoustic emissions in cochlea-specific barttin KO mice, indicating that outer hair cells (OHCs) had lost their ability to mechanically amplify cochlear sound. Because the loss of OHC function was already complete at the onset of hearing, the subsequent degeneration of hair cells did not further impair hearing. The extent of hearing loss (~60 dB) was compatible with a complete loss of OHC function,



although the decrease in EP should also reduce the transduction current of inner hair cells which directly provide the sound-encoding electrical signals for transmission to the brain.

Interestingly, a common polymorphism in the *CLCNKB* gene (present in 20 to 40% of the European population) leads to a strong, roughly 20-fold augmentation of ClC-Kb/barttin currents [95]. If basolateral chloride efflux was rate-limiting in salt absorption, this polymorphism (leading to a T481S amino-acid exchange) might increase NaCl reabsorption, thereby leading to hypertension. Indeed, an initial study found a slight correlation with higher blood pressure in individuals hetero- or homozygous for this polymorphism [96], but several follow-up studies failed to see an effect on blood pressure in other cohorts [97–99]. The same polymorphism might possibly also increase the endocochlear potential by stimulating potassium secretion by stria marginal cells (endocochlear  $[K^+]$  not being able to rise further for osmotic reasons). Indeed, the same group that associated the T481S polymorphism with hypertension [96] also found a correlation with increased sensitivity of hearing [100]. This result requires replication in other cohorts.

#### 2.4. Loss of ClC-5 $Cl^-/H^+$ -exchanger: impaired endocytosis leads indirectly to kidney stone

In contrast to members of the first branch of the ClC gene family, which encodes plasma membrane  $Cl^-$  channels, ClC-5 is found predominantly on endosomes [101] and functions as an electrogenic  $Cl^-/H^+$ -exchanger with a likely stoichiometry of  $2Cl^-/1H^+$  [102,103]. At least upon heterologous expression, a small proportion of ClC-5 is located also at the plasma membrane [101], which has greatly helped in determining its transport properties. The transport rate of ClC-5, like those of its close homolog ClC-4 and probably also ClC-3 [102,104,105], is strongly voltage-dependent and above background only at potentials more positive than roughly +20 mV [106,107]. Because the plasma membrane and probably also intracellular membranes do not display such voltages, it remains a mystery how ClC-5 mediates significant transport in physiological settings [3].

ClC-5 is mainly expressed in renal and intestinal epithelia, where it is found in endosomes in the apical, but not basolateral cell pole [101,108]. It was co-localized with endocytosed protein both in transfected and native cells, and was enriched in the enlarged early endosomes that are generated by transfection of a rab5 mutant deficient for GTPase activity [101]. In the kidney, ClC-5 is most highly expressed in  $\alpha$ - and  $\beta$ -intercalated cells of the distal nephron, followed by the proximal tubule (PT) where it is prominently expressed in a vesicular region below the highly infolded, apical brush border membrane [101,109]. Depending on the conditions of tissue fixation, a weaker ClC-5 staining is also observed in the brush border, suggesting that it may recycle from endosomes over the plasma membrane. In addition, ClC-5 is also weakly expressed in the thick ascending limb of Henle's loop [110].

ClC-5 is mutated in Dent's disease [111,112], a rare X-linked kidney stone disorder that is associated with the loss of low molecular weight proteins into the urine. Whereas proteinuria is a constant feature of Dent's disease, hypercalciuria, hyperphosphaturia, and in particular kidney stones and nephrocalcinosis are more variable symptoms of that syndrome [113]. The selective loss of low molecular weight proteins, which can be filtered into the primary urine and are normally endocytosed by the proximal tubule, points to a defect in proximal tubular endocytosis – a hypothesis bolstered by the localization of ClC-5 on apical endosomes of the PT. The observation that patients with Dent's disease often present with glycosuria or aminoaciduria points to a more generalized dysfunction of the proximal tubule, which is often referred to as Fanconi syndrome.

In order to clarify the pathological mechanisms leading to the symptoms of Dent's disease, two groups generated constitutive ClC-5 knock-out mouse models [114,115]. Both models displayed low

molecular weight proteinuria and hyperphosphaturia, but only the KO mouse from the Guggino lab [115], and not the mouse from the Jentsch lab [114], displayed hypercalciuria and interstitial calcium accumulation in the kidney. *In vivo* endocytosis experiments with the Jentsch mouse model showed that the disruption of ClC-5 severely reduced receptor-mediated and fluid-phase endocytosis, as well as the endocytosis of certain plasma membrane proteins like NaPi-2a and NHE3, in a cell-autonomous manner [114]. Furthermore, the expression of megalin, an apical recycling receptor that is responsible for the endocytosis of a broad range of proteins and other substances, was reduced cell-autonomously as shown in chimeric tubules that contain ClC-5 positive and negative cells side by side [114]. This decrease in megalin, which is expected to impair receptor-mediated endocytosis even further, was tentatively attributed to a defect of its recycling to the brush border. Indeed, a later study found decreased brush-border levels of megalin and of its co-receptor cubilin in PTs lacking ClC-5 [116]. In contrast to megalin KO mice, the morphology of the endocytic compartments were not changed when examined by EM. However, the lysosomal accumulation of cathepsin B was impaired in ClC-5 KO mice, indicating that a significant proportion of the degradative enzyme reaches lysosomes by apical endocytosis [117].

Why does the lack of ClC-5 impair endocytosis? It is well known that endosomes, lysosomes, and also vesicles of the secretory pathway are acidified by V-type  $H^+$ -ATPases. These pumps are electrogenic and would build up a vesicle-inside positive potential that would inhibit further pumping, unless there is a conductive pathway for another ionic species that dissipates that potential. As many studies have indicated that chloride is needed for vesicular acidification, this finding suggested that ClC-5 provides such a conductive pathway. Indeed, renal cortical endosomes from ClC-5 KO mice showed less ATP-dependent acidification *in vitro* when compared to WT endosomes [114,118], as did early/recycling (but not late) endosomes studied in renal proximal tubule cells in culture [119] – a finding perfectly compatible with the localization of ClC-5 to early and recycling endosomes. An acidic luminal pH is required for several aspects of endosome function, including endosomal carrier formation [120] and the binding of the regulator proteins ARNO and Arf6 [121,122]. Furthermore, impairing endosomal acidification inhibited endosomal trafficking in several studies [123–125]. Thus, defective acidification may underlie the broad defect in endocytosis owed to a loss of ClC-5. This argument is still valid with the discovery that ClC-5 is a  $Cl^-/H^+$ -exchanger and not a  $Cl^-$  channel, as thought previously. The direct coupling of chloride to pH-gradients, however, suggests that also luminal chloride may play a role [3].

If the lack of ClC-5 causes a defect in endocytosis and thereby proteinuria, how then to explain hyperphosphaturia, hypercalciuria and kidney stones? We have proposed that these are secondary effects of impaired endocytosis and metabolism of calciotropic hormones [114,118,126]. Both parathyroid hormone and vitamin D (bound to its binding protein) are filtered into the primary urine and are normally endocytosed in a megalin- and ClC-5-dependent manner in the proximal tubule [127,128]. The disruption of this process in the absence of ClC-5 leads to higher than normal levels of PTH in later portions of the PT. The resultant excessive stimulation of apical PTH receptors will then lead to the endocytosis and degradation of NaPi-2a, as has been observed in ClC-5 KO mice [114]. Since NaPi-2a is the main pathway for phosphate uptake in the PT, hyperphosphaturia ensues.

The proposed pathway for hypercalciuria is more complex. The proximal tubule is the main site for converting the inactive precursor 25(OH)VitD<sub>3</sub> to the active hormone 1,25(OH)<sub>2</sub>VitD<sub>3</sub>. The enzyme carrying out this conversion,  $\alpha$ -1-hydroxylase, is stimulated by PTH and is indeed upregulated in ClC-5 KO kidneys [114,118,126]. Another factor contributing to its upregulation is certainly the decreased apical endocytotic uptake of VitD (active and inactive forms) into proximal tubular cells, because its transcription is repressed by 1,25(OH)<sub>2</sub>VitD<sub>3</sub>. The upregulation of the hydroxylase would predict increased serum

levels of  $1,25(\text{OH})_2\text{VitD}_3$ , should there be no urinary loss of  $1,25(\text{OH})_2\text{VitD}_3$  and a decreased uptake of the precursor into PT cells owed to the loss of CIC-5. Thus, there will be two opposing mechanisms influencing  $1,25(\text{OH})_2\text{VitD}_3$  levels. This active hormone stimulates intestinal reabsorption of  $\text{Ca}^{++}$ , which then in turn would have to be eliminated by the kidney, resulting in hypercalciuria. Indeed, moderately elevated serum levels of  $1,25(\text{OH})_2\text{VitD}_3$  were found in patients with Dent's disease [129] and the hypercalciuric CIC-5 KO mouse from Guggino's lab [115,130]. By contrast, our CIC-5 KO mouse, which lacks hypercalciuria, displayed decreased levels of  $1,25(\text{OH})_2\text{VitD}_3$  [114,126]. By explaining hypercalciuria and kidney stones as being the result of a complicated cascade of events that results from a primary defect in endocytosis, this well-supported hypothesis is able to explain the clinical variability of Dent's disease. However, we would like to mention that intestinal  $\text{Ca}^{++}$  reabsorption may not be increased in Guggino's KO mouse [130,131].

Genome-wide expression analysis of CIC-5 KO kidneys revealed that target genes of  $1,25(\text{OH})_2\text{VitD}_3$  are downregulated in the PT. This can be explained by the impaired apical uptake of the vitamin that leads to a reduced concentration VitD in the cell. By contrast, these genes are upregulated in more distal segments, probably because the decreased VitD uptake in the PT entails an increased luminal concentration in those segments. This concept could be extended to target genes of retinoic acid [126]. Studies of CIC-5 KO mice have thereby led to the novel principle that local changes in the luminal concentration of hormones are important for renal pathology and physiology.

The mechanism by which the loss of CIC-5 affects tubular reabsorption of other substrates (like amino-acids or glucose) is less clear. Rather unexpectedly, however, it is not the abundance of apical, but basolateral glucose transporters that was changed in the Guggino mouse model [132].

A much less pronounced proteinuria was described recently for mice disrupted for CFTR, the cAMP-activated chloride channel affected in cystic fibrosis [133]. Similar to CIC-5, CFTR was detected in PT endosomes, with its expression being highest in the S3 segment. The rather selective proteinuria was attributed to a decrease in the expression of the endocytotic co-receptor cubulin [133].

It should be mentioned that Dent's disease can also be caused by mutations in *ORCL1*, which encodes a phosphatidylinositol 4,5-bisphosphate 5-phosphatase [134]. Because phosphoinositides regulate various steps of membrane trafficking [135], altered ORCL1 function may cause endocytotic trafficking defects independent from changes in endosomal pH or  $\text{Cl}^-$  concentration. It remains unclear why some mutations on ORCL1 cause Dent's disease, whereas the majority of mutations in the same gene lead to the much more complex Lowe oculocerebrorenal syndrome [136].

## 2.5. CIC-7/*Ostm1*: osteopetrosis and lysosomal storage

CIC-7 is broadly expressed and may be found, to different extent, probably in all tissues [78]. CIC-7 is the only member of the CLC family that is prominently expressed on lysosomes, as demonstrated by co-localization in immunocytochemistry, immuno-gold EM, and subcellular fractionation [137,138]. Because CIC-7 displays the 'proton glutamate' that is typical for CLC  $\text{Cl}^-/\text{H}^+$ -exchangers, it was believed that it functions as an exchanger rather than a channel [3]. Indeed, isolated liver lysosomes display  $2\text{Cl}^-/\text{H}^+$  exchange [139]. CIC-7 needs *Ostm1*, a highly glycosylated type I transmembrane protein, as  $\beta$ -subunit [140]. In native tissue, the CIC-7 protein is unstable without *Ostm1* and vice-versa [140]. The lack of plasma-membrane expression of CIC-7/*Ostm1* has severely hindered its biophysical characterization.

An explorative knock-out of CIC-7 in mice [137] revealed its importance for lysosomal function and for bone resorption by osteoclasts. Mice lacking CIC-7 displayed severe osteopetrosis, retinal

degeneration [137], as well as lysosomal storage disease in the central nervous system (CNS) and kidney proximal tubules [138]. CIC-7 was found to be highly expressed in the ruffled border of osteoclasts [137] that secretes protons into the resorption lacuna. This fits well to the lysosomal localization of CIC-7, because the ruffled border is generated by the exocytotic insertion of lysosomal membranes that also contain the V-type  $\text{H}^+$ -ATPase. An acidic pH in the resorption lacuna is necessary for the dissolution of the inorganic bone material, as well as for the activity of lysosomal enzymes that are secreted into the resorption lacuna and that degrade the organic bone matrix. Osteoclasts isolated from CIC-7 KO mice failed to acidify their resorption lacuna when cultured on ivory slices and, in contrast to WT osteoclasts, could not chew holes into the bone surrogate [137]. It was suggested that CIC-7 provides a conductive pathway for the  $\text{H}^+$ -ATPase in the ruffled border, similar to the role of CIC-5 in endosomes that was discussed above. On the other hand, CIC-7 might also be important for the exocytotic insertion of lysosomal membranes into the ruffled border. In fact, this membrane did not show the highly infolded, large surface as in WT osteoclasts when examined by electron microscopy [137].

The finding that loss of CIC-7 causes osteopetrosis in mice prompted us to search for mutations in the human *CLCN7* gene in patients with recessive, malignant infantile osteopetrosis (autosomal recessive osteopetrosis, ARO). Indeed, one patient was a compound heterozygote for *CLCN7* mutations [137]. In the meantime, more than 30 different mutations in *CLCN7* have been identified in humans with osteopetrosis. This includes mutations in patients with autosomal dominant osteopetrosis (Albers-Schönberg disease) [141]. This dominant form of the disease is much less severe, with osteopetrosis sometimes becoming apparent only in adult life. In contrast to ARO (see below), there are no neurological symptoms. Taking into account the dimeric structure of CLC proteins and findings with CIC-1 in recessive and dominant myotonia, one expects that those patients still have 25% fully functional WT/WT CIC-7 dimers. A broader role for CIC-7 in bone metabolism is suggested by the finding that *CLCN7* polymorphisms were associated with variations in bone mineral density [142,143].

Other cells, including neurons, also prominently express CIC-7. Similar to many patients with osteopetrosis, CIC-7 KO mice display retinal degeneration. Whereas in many forms of osteopetrosis retinal degeneration results from optic canal narrowing and subsequent compression of the optic nerve owed to the osteopetrotic process, their retinal degeneration is a direct consequence of the CIC-7 loss in the retina [137]. In fact, the retina of CIC-7 KO mice, whose osteopetrotic phenotype was rescued by the transgenic expression of CIC-7 in osteoclasts, degenerated with the same time course [138].

CIC-7 KO mice also display neurodegeneration with the typical hallmarks of lysosomal storage disease [138]. Electron microscopy detected electron-dense storage material in neuronal cell bodies throughout the brain. At 4–6 weeks of age (the maximum life span of *Clcn7*<sup>-/-</sup> mice), there was conspicuous neuronal cell loss in the CA3 region of the hippocampus and in the cerebral cortex. The morphology of deposits, the accumulation of subunit c of the mitochondrial ATP synthase, as well as several biochemical markers strongly resembled findings with human neuronal ceroid lipofuscinosis (NCL) [138]. CIC-7 KO mice, in which osteopetrosis was selectively rescued by transgenic expression of CIC-7 in osteoclasts, survived only a few weeks longer than the total KO. Neurological symptoms suggested that they died from a dysfunction of the CNS. Whereas early reports suggested that a small subset of patients with severe osteopetrosis also suffered from neurodegeneration (as reviewed in [144]), only recently patients with confirmed *CLCN7* mutations were shown to have CNS abnormalities [145]. These findings indicate that whereas bone marrow transplantation may cure the osteopetrosis of patients with a total loss of CIC-7 function, those patients will still suffer from blindness and severe CNS degeneration.

The lysosomal storage of CIC-7 KO mice fits well to CIC-7 being the main CLC protein of lysosomes [137,138]. Because disruption of CIC-5 impaired endosomal acidification [118,119], disruption of CIC-3 reduced acidification of endosomes [146] and synaptic vesicles [7], and because the resorption lacuna of *Clcn7*<sup>-/-</sup> mice was not acidified [137], we suspected that the lysosomal pathology of CIC-7 KO mice was caused by a failure of lysosomal acidification. However, careful ratiometric pH measurements of hundreds of lysosomes from neurons and fibroblasts showed that steady-state lysosomal pH of *Clcn7*<sup>-/-</sup> cells was undistinguishable from WT controls [138,140]. A later study [139] using a single siRNA to mildly reduce CIC-7 levels and a single negative control siRNA seems to contradict our results. However, those authors did not use ratiometric pH measurements, but rather measured total lysotracker fluorescence [139]. Instead of reporting pH, the reported changes in fluorescence may equally well indicate differences in lysosome numbers or sizes, both of which were not determined. A further reason for concern is the increase in lysotracker fluorescence (interpreted as hyperacidification) measured in cells transfected by a single control siRNA that should rather have no effect on pH [139].

As shortly mentioned above, CIC-7 needs the small Ostm1 protein as  $\beta$ -subunit [140]. Ostm1 was identified by Vacher et al. [147] as the gene mutated in *grey lethal* mice, a spontaneous osteopetrotic mouse model. *OSTM1* mutations can also be found in some patients with severe infantile recessive osteopetrosis [147,148]. Both *grey lethal* mice and *Clcn7*<sup>-/-</sup> mice have grey fur in an *agouti* genetic background, possibly pointing to a defect in melanosomes (which share some features with lysosomes). The similarity in phenotypes prompted us to investigate whether there is some functional connection between these two proteins. We showed that Ostm1, just like CIC-7, localizes to lysosomes and the ruffled border of osteoclasts [140]. Ostm1 needs CIC-7 to travel to lysosomes. Ostm1 could be co-precipitated with CIC-7 and vice-versa, suggesting that Ostm1 is a tightly associated ancillary  $\beta$ -subunit of CIC-7 [140]. Importantly, CIC-7 protein levels were reduced to about 5% of WT levels in *grey lethal* mice, and Ostm1 levels were similarly down in CIC-7 KO tissues. Hence the phenotype of *grey lethal* mice, which similar to *Clcn7*<sup>-/-</sup> mice show lysosomal storage in addition to osteopetrosis, may be entirely attributed to the instability of the CIC-7 when it lacks its  $\beta$ -subunit [140].

### 3. CFTR, a cAMP-activated Cl<sup>-</sup> channel, and cystic fibrosis

Cystic fibrosis (CF) is a multisystemic disease that is characterized by abnormal salt and water transport across epithelia. Among other symptoms, this leads to an obstruction of ducts of mucous glands and altered composition of fluid films covering lung epithelia. A broad spectrum of symptoms is found in CF. Benign forms of CF can be nearly asymptomatic, being diagnosed only in adulthood and affecting just a single organ. For instance, some male CF patients are first diagnosed during male infertility screening. Virtually all (96–98%) CF males are infertile because of obstructive azoospermia due to congenital atrophied distal epididymis, vas deferens, or seminal vesicles. In contrast, fertility in female CF patients is only slightly reduced compared to healthy women. On the opposite side of the spectrum, more severe forms of CF give rise to a multiorgan disease which, in addition to male infertility, leads to severe respiratory impairment and may include pancreatic insufficiency, liver disease and gut obstruction in newborns (meconium ileus). Impairment of intestinal ion and water transport may lead to nutrient malabsorption and malnutrition. In the lung, a reduction of the Airway Surface Liquid (ASL) thickens the mucus and impairs mucociliary movement which facilitates bacterial infection, inflammation and finally leads to tissue destruction [149]. In the pancreas, duct obstruction is followed by cystic dilatation, fibrosis and atrophy of the gland [150]. Hepatobiliary complications (stones, bile duct stricture) might result in biliary cirrhosis, portal hypertension and eventually liver failure [151,152].

CF is the most frequent hereditary lethal disease affecting the Caucasian population, with an incidence of approximately 1:3000. The incidence in other ethnic groups is much less prominent (in Hispanics 1:8000) and is rare in the native African and Asian populations (estimated 1:50,000). CF, as an autosomal recessive disease, is diagnosed on the premise of specific clinical symptoms (e.g. recurrent pneumonia or pancreatic insufficiency), evidence of familial history, and/or presence of two CF-causing mutations (in *trans*) [152]. The course of disease is variable. The median age at diagnosis is in the first 6 months of life. Median survival ranges between the second and third decade and is strongly dependent on the severity of pulmonary and/or gastrointestinal disease. Ninety percent of CF deaths are from pulmonary disease. At birth, CF patients have nearly normal lungs. However, subsequent pulmonary infections may lead to bacterial colonization promoted by the thickened mucus and impairment of mucociliary clearance. Airway infection leads to persistent inflammatory response that may destroy small airways leading to bronchiectasis, emphysema or pneumothorax. Survival has improved, thus far, through aggressive management of pulmonary, pancreatic, and intestinal complications. Indeed, life expectancy for patients with milder forms of CF with appropriate medical treatment may approach that of unaffected people. Despite advances in treatment, there is no cure for CF.

For clinical diagnosis, a chloride concentration of  $\geq 60$  mM in sweat is considered discriminating for CF. The altered electrolyte levels in the sweat of CF patients indicated that the primary defect in CF might be an impaired chloride transport. In 1983, Quinton identified a cAMP-regulated chloride conductance in the sweat ducts of CF patients that was decreased/defective compared to normal patients [153]. Alternatively, CF can be diagnosed by electrophysiological measurement of nasal potential difference (NPD) to assess amiloride-inhibitable Na<sup>+</sup> transport and cAMP-mediated Cl<sup>-</sup> transport. Specifically, in normal airways epithelium, baseline NPD is approximately -30 mV and upon amiloride perfusion becomes partially (~60%) depolarized. In CF epithelium, basal NPD is close to -60 mV largely due to an increase in the amiloride-sensitive component [154,155]. In airway epithelial CF cells, the decrease in Cl<sup>-</sup> current is accompanied by an increased Na<sup>+</sup> conductance [156]. In 1989, the causative gene was identified on chromosome 7 by positional cloning [157,158]. It codes for a protein of 1480 amino acids, the CF Transmembrane Conductance Regulator or CFTR. CFTR is expressed on the luminal surface of several epithelia including respiratory pseudostratified epithelia, in acini of submucosal glands, in enterocytes and goblet cells (with more abundant expression in duodenum and jejunum, and decreasing in more distal parts of intestine), in pancreatic duct, biliary tree and vas deferens.

CFTR is a member of the ABC transporters family of proteins, integral membrane proteins topologically characterized by two homologous domains, each of which is composed of a membrane-spanning domain (MSD) followed by a cytoplasmic nucleotide-binding domain (NBD) [159]. Akin to other ABC family members, CFTR contains two ATP binding sites. Only one of them has hydrolytic ATPase activity. Both binding sites are located at the interface between the two NBDs, with each NBD contributing residues for ATP binding [160,161]. Since ABC transporters generally are active transporters that use the energy of ATP hydrolysis to pump a variety of different substances, it came as a surprise that CFTR functions as a chloride channel [162,163]. The gating of the CFTR pore seems to be regulated by ATP binding and hydrolysis (while channel opening is regulated by ATP binding and/or events secondary to ATP binding, the rate of closure depends on ATPase activity) [164–166]. In addition, CFTR contains a unique regulatory (R) domain located between NBD1 and MSD2. Phosphorylation by cAMP-dependent protein kinase A of various residues in this domain mediates the activation of the CFTR channel by cAMP (for a review on CFTR structure and function, see [167]).

As mentioned above, the mucosa of proximal and distal airways is covered by Airway Surface Liquid (ASL), which is a first barrier against



inhaled pathogens. ASL is composed of a periciliary liquid layer (PCL) surrounding the cilia of epithelial cells, and on top of that a mucin-rich mucous layer. In order to optimize ciliary beating and mucus clearance, ASL volume and ionic composition must be tightly regulated [168]. The transepithelial flux of  $\text{Cl}^-$  and  $\text{Na}^+$  will determine the passive movement of water that follows the osmotic gradient. In secretory cells, such as in serous cells of submucosal glands or colonic crypt cells, apical CFTR chloride channels form the exit pathway for  $\text{Cl}^-$  which is taken up by the basolateral  $\text{NaK2Cl}$  cotransporter. The latter is driven by the  $\text{Na}^+$  gradient established by the basolateral  $\text{Na,K-ATPase}$  and needs  $\text{K}^+$  recycling via basolateral  $\text{K}^+$  channels [169,170]. In lung surface epithelia, there is a delicate balance between CFTR-mediated  $\text{Cl}^-$  secretion and  $\text{Na}^+$ -reabsorption mediated by the epithelial  $\text{Na}^+$  channel ENaC (for a recent review, see [149]). Significant counterion transport occurs paracellularly in this 'leaky' epithelium. Therefore, activation of CFTR can lead to  $\text{NaCl}$  secretion ( $\text{Na}^+$  moving paracellularly) and activation of ENaC to  $\text{NaCl}$  absorption (paracellular movement of  $\text{Cl}^-$ ). There may also be a  $\text{Ca}^{++}$ -activated  $\text{Cl}^-$  channel in the apical membrane. These ion channels are regulated by extracellular ATP and adenosine, which activate intracellular signalling cascades through  $\text{P2Y}_2$  and  $\text{A}_{2b}$  receptors. The situation is further complicated by the fact that ENaC may be inhibited by CFTR involving poorly understood and controversial mechanisms [171–175]. Conversely, an activation of ENaC by extracellular proteases is well established and may play an important role in CF [176–178].

According to the hydration hypothesis, airway surface dehydration is responsible for diminished mucociliary clearance, leading to higher susceptibility to viral and bacterial infections and thereby to CF lung disease ([179]; for a recent review, see [149]). As discussed above, failure to adequately hydrate mucus airway epithelia in CF could arise from either the absence/decreased capacity to secrete  $\text{Cl}^-$  or/and from the continuously upregulated  $\text{Na}^+$  absorption. In addition to the role of CFTR  $\text{Cl}^-$  channel activity in salt/water homeostasis in exocrine tissues, CFTR also has an important role in  $\text{HCO}_3^-$  secretion: in pancreas duct cells, secretin and other gastrointestinal hormones induce  $\text{HCO}_3^-$  secretion and stimulate the apical  $\text{Cl}^-/\text{HCO}_3^-$  exchanger whose exchange rate depends on  $\text{Cl}^-$  efflux by CFTR. In bronchial epithelial cultures from CF patients, ASL becomes acidified, which in turn may alter normal processing of mucins [180].

More than 1600 different CF associated mutations in *CFTR* have been reported. These mutations have been classified into functional categories: Class I mutations abrogate the synthesis of a functional protein e.g. by premature stop codons or frame shifts, while Class II mutations lead to biosynthetic processing defects, intracellular protein retention and degradation. A prototype of Class II mutation is a single codon deletion  $\Delta\text{F508}$  in the NBD1 domain, reported in approximately 70% of Caucasian mutant alleles [181]. Different studies have revealed that WT CFTR is subject to an extensive quality control system during its biosynthesis and that a large fraction of the protein is degraded before reaching the plasma membrane.  $\Delta\text{F508}$  CFTR fails to reach the plasma membrane because it is retained in the endoplasmic reticulum, as biochemically evident from incomplete glycosylation [167]. Lowering the temperature, however, leads to a partial expression in the plasma membrane where it was shown to be able to mediate  $\text{Cl}^-$  currents that may have altered biophysical properties [182,183]. Class I and II mutations prevent sufficient CFTR  $\text{Cl}^-$  channel expression at the plasma membrane and, when present on both alleles, are associated with multiorgan disease including infertility, pancreatic deficiency and progressive pulmonary disease. Class III mutants result from missense or deletion mutations that alter channel gating, usually rendering channels insensitive to activation. This class of mutations is generally also associated with severe disease symptoms. In contrast, Class IV mutant channels display normal protein expression levels, respond to cAMP stimulation, but have reduced chloride conductance. The partial loss of channel function is generally associated with a milder disease. A correlation between the

level of functional CFTR and organ susceptibility has been proposed, with lung and pancreas being the least sensitive organs (CFTR channel activity has to be reduced to <1% to cause disease), sweat duct (reduction to <4%) and vas deferens being the most sensitive (<10%) [184,185].

Different CF mouse models have been generated to mimic CF by producing truncated CFTR [186,187] or by deleting F508 to mimic the most common human allele [188,189]. The upper airway epithelia exhibited marked decrease in cAMP-mediated  $\text{Cl}^-$  current and decreased  $\text{Na}^+$  absorption. Surprisingly, however, these mice lacked an overt pulmonary phenotype, but showed high early postnatal lethality owed to intestinal obstruction similar to the meconium ileus that may be observed in CF infants. Recorded basal potential difference across tracheal epithelia in CF and normal mice had similar values, in contrast to NPD values in CF and normal humans, thus suggesting that an alternate  $\text{Cl}^-$  current was capable of compensating the lack of CFTR in murine CF airways [190]. Nevertheless, infection studies with *Pseudomonas aeruginosa* in CF mice produced an increased inflammatory response that culminated in higher proportion of weight loss and death compared to WT animals [191]. More recently, a new CF model in pigs has been generated that developed meconium ileus, degenerative exocrine pancreas and liver abnormalities, but so far no alterations in lungs or in vas deferens have been reported [192]. Nonetheless, because the anatomy and physiology of the lung of pigs more closely resemble the human lung than that of mice, this pig CF model promises to yield important insights into human CF pathology. The lung of CF pigs has only been investigated in young animals, and progressive age-related CF-like pathology may become evident later in their airways and reproductive tract like in some mouse CF models [193].

Given that the CFTR  $\text{Cl}^-$  channel is absent or inactive in CF, it has been suggested that alternative  $\text{Cl}^-$  pathways may partially compensate for mutant CFTR. The voltage gated  $\text{Cl}^-$  channel  $\text{ClC-2}$  was initially suggested to mediate  $\text{Cl}^-$  secretion across apical membranes of airway epithelia [52]. However, in surface epithelia of mouse colon,  $\text{ClC-2}$  is exclusively detectable in the basolateral membrane [49,65,68], and mice with a targeted disruption of both CFTR and  $\text{ClC-2}$  did neither show lung nor pancreatic disease. Mice homozygous for the  $\Delta\text{F508}$  mutation survived even better with an additional disruption of the  $\text{ClC-2}$  gene [65]. In a CFTR-deficient pancreatic duct cell line,  $\text{Cl}^-/\text{HCO}_3^-$  exchange is supported by  $\text{Ca}^{++}$ -activated chloride channels as a recycling pathway [194], and the latter channels remain good candidates for substituting CFTR in electrodiffusive chloride transport across apical membranes of epithelia. As discussed in more detail below, TMEM16A has recently been identified by three independent groups as a protein that recapitulates the properties of native epithelial  $\text{Ca}^{++}$ -activated  $\text{Cl}^-$  channels [195–197].

Major therapeutical advances during the last 30 years have increased the median life expectancy of CF patients to 30–40 years. These therapies are based on improving nutrition and on early and aggressive pharmacological intervention to treat the lung disease, which ultimately determines the survival of CF patients. This includes treatment with hypertonic saline, antibiotics, bronchodilators, anti-inflammatory drugs, mucolytic agents, and chest physiotherapy. Gene replacement appeared to hold great promise for cystic fibrosis as lung epithelia might be easily accessible by inhalation, at least in the absence of obstructing mucus. However, even after many years of research, there remain many obstacles to be overcome. An Adeno-Associated Virus (AAV) carrying CFTR cDNA has proved effective in primary cultures and macaques [198,199], although a T-cell response was observed in mice [200]. In addition, two clinical studies using AAVs have produced different results regarding the effectiveness of treatment and beneficial outcome in CF patients [201,202]. Nevertheless, further improvements need to be obtained regarding efficient delivery and sustained expression over the lifespan of airways epithelial cells. Another pharmacological strategy which could benefit



specific CF patients with truncated CFTR (approx. 10%) is the use of amino glycoside antibiotics such as gentamycin that promote low-frequency read-through translation of transcripts. A clinical study with gentamycin applied to the nasal mucosa of patients carrying the W1282X allele, a truncation in NBD2 but able to produce barely functional  $\text{Cl}^-$  channels, decreased the transepithelial potential difference and yielded full-length CFTR [203]. This strategy was, however, unsuccessful in patients with low level transcripts of CFTR. For patients carrying the  $\Delta\text{F508}$  mutation allele (which yields functional CFTR that is retained in the ER), diverse high-throughput assays have yielded either CFTR potentiators or ER correctors that are capable to allow CFTR to escape to the plasma membrane (for a review, see [204]). At present, some of these compounds undergo clinical trials in CF patients. An alternative approach proposes to pharmacologically modulate alternative epithelial ion channels like  $\text{Ca}^{++}$ -activated  $\text{Cl}^-$  channels or the sodium channel ENaC. An activation of the former channels may provide an alternative pathway for apical  $\text{Cl}^-$  transport. ENaC activation, however, contributes to CF lung pathology by promoting  $\text{Na}^+$  reabsorption and consequently a reduction of the ASL. Indeed, a transgenic mouse model overexpressing the  $\beta\text{ENaC}$  subunit displays CF-like symptoms [205]. On the other hand, also a pathological reduction of ENaC expression in the lung is deleterious. CF-like symptoms were observed in a patient carrying a mutation in the ENaC  $\beta$ -subunit that causes a partial loss of function [206], and mice with reduced ENaC  $\beta$ -subunit expression displayed impaired lung fluid clearance [207]. The activation of ENaC in CF might be a consequence of a lack of a poorly characterized, direct interaction with CFTR, and/or may be owed to a proteolytic activation of ENaC through extracellular proteases that are a by-product of airway inflammation. In either case, inhibition of ENaC is a promising therapeutic strategy. Although therapeutic effects of ENaC inhibitors, such as amiloride or more potent and long-lived derivatives, were initially minimal because of their unfavorable pharmacodynamic properties [208], more suitable derivatives of amiloride are being developed [209]. Inhibition of cell surface serine proteases to avoid ENaC cleavage and its subsequent activation might also be promising [210].  $\text{P2Y}_2$  agonists are also being used in clinical trials since they both inhibit ENaC and activate  $\text{Ca}^{++}$ -activated  $\text{Cl}^-$  channels, thus improving hydration of ASL in CF [211]. These therapeutic approaches are currently more promising than gene therapy.

#### 4. $\text{Ca}^{++}$ -activated chloride channels

The molecular identity of  $\text{Ca}^{++}$ -activated  $\text{Cl}^-$  channels has been obscure and controversial for many years. The discovery of bestrophins, which are *bona fide*  $\text{Cl}^-$  channels in addition to having other functions, and in particular the identification of TMEM16A and B as excellent candidates for typical  $\text{Ca}^{++}$ -activated  $\text{Cl}^-$  channels that have been studied biophysically for a long time, has changed that picture. Two human diseases could be associated to mutations in genes that may encode  $\text{Ca}^{++}$ -activated  $\text{Cl}^-$  channels. At this point, both diseases should rather be considered as putative channelopathies.

##### 4.1. Bestrophin and Best's macular dystrophy — a chloride channelopathy?

Best's vitelliform macular dystrophy is an autosomal dominant disorder in which lipofuscin accumulates in and close to the retinal pigment epithelium. It shows a characteristic feature in electroretinograms, an absence of a slow wave that is believed to be a response of the pigment epithelium that underlies the retina in response to the light-induced electrical response of photoreceptors [212]. It is thought that this wave represents the activation of  $\text{Ca}^{++}$ -activated  $\text{Cl}^-$  currents in basolateral membranes of retinal pigment epithelial cells [213]. This abnormal retinogram is a consistent feature of the disease, whereas the progressive loss of visual acuity is much more variable. The gene

underlying Best's macular dystrophy was identified by positional cloning [214,215] in 1998 and shown to encode an integral membrane protein with most likely four transmembrane spans [216,217]. This gene, now named bestrophin 1, defines a family of related bestrophins with four members in mammals and at least 25 in the nematode *C. elegans*. As bestrophin1 was localized to basolateral membrane of the pigment epithelium [218], it seemed reasonable to suspect that the gene product functions as a  $\text{Ca}^{++}$ -activated  $\text{Cl}^-$  channel. Indeed, heterologous expression yielded  $\text{Cl}^-$  currents that could be activated by raising intracellular  $\text{Ca}^{++}$ , and, reassuringly, expression of other isoforms (e.g. from *Drosophila*) gave currents that differed in kinetics from human Best1 [216]. However, the  $\text{Ca}^{++}$ - and voltage-dependence of these currents did not match those observed in most native cells. Initially several laboratories failed to observe currents upon expression of bestrophin genes, and other functions of bestrophins were described. Bestrophin was reported to interact with protein phosphatase 2A [219] and to modulate  $\text{Ca}^{++}$  channels [220,221], and the light peak of the electroretinogram may rather depend on  $\text{Ca}^{++}$  channels modulated by bestrophin 1 [222]. The differences in biophysical properties of currents elicited by different isoforms or mutants, however, strongly suggests that bestrophins also act as  $\text{Cl}^-$  channels [216,223–225] (for a review, see [226]). The mechanism by which mutations in bestrophin1 lead to Best's vitelliform macular dystrophy, however, remains obscure [226], and the disruption of bestrophin1 in mice did not abolish  $\text{Ca}^{++}$ -activated  $\text{Cl}^-$  currents in the retinal pigment epithelium [222]. Thus, even though there is strong evidence that bestrophins can function as  $\text{Cl}^-$  channels, doubts remain as to whether Best's macular dystrophy is directly due to a loss of chloride channel activity.

##### 4.2. Gnathodiaphyseal dysplasia, a TMEM16 $\text{Cl}^-$ channel disease?

A family of putative  $\text{Ca}^{++}$ -activated chloride channels was recently discovered by expression cloning of a *Xenopus* TMEM16A orthologue [196] or in approaches involving bioinformatic analysis [227,228]. In contrast to bestrophins, currents mediated by TMEM16A and B resemble typical  $\text{Ca}^{++}$ -activated  $\text{Cl}^-$  currents found endogenously in many cells. In humans, there are 10 different TMEM16 genes, all of which encode integral membrane proteins with about 8 transmembrane domains. Upon heterologous expression, TMEM16A yielded  $\text{Ca}^{++}$ -activated chloride currents [196,227,228], as did its *Xenopus laevis* ortholog and TMEM16B [196]. As expected from the assumed role of  $\text{Ca}^{++}$ -activated  $\text{Cl}^-$  channels in the salivary gland, siRNA against TMEM16A impaired pilocarpine-induced salivary flow [228], and endogenous  $\text{Ca}^{++}$ -activated chloride currents were downregulated by siRNAs [227].

Interestingly, another member of the gene family, TMEM16E (also known as GDD1) is mutated in human autosomal dominant gnathodiaphyseal dysplasia [229]. This multi-faceted syndrome is associated with bone dysplasia and fragility, as well as purulent osteomyelitis of the jaws. Intriguingly, the only mutations found in patients are (different) missense mutations in a cysteine residue that is highly conserved in this gene family [229]. The TMEM16E protein is most highly expressed in cardiac and skeletal muscle tissues, as well as in growth-plate chondrocytes and osteoblasts [230]. It is present on intracellular vesicles the identity of which could not be determined unambiguously [230]. Although a reasonable working hypothesis that would link impaired  $\text{Ca}^{++}$ -activated  $\text{Cl}^-$  channels to the various symptoms of gnathodiaphyseal dysplasia is lacking, the existence of such a relationship can certainly not be ruled out — a case in point being osteopetrosis owed to a disruption of the lysosomal  $\text{ClC-7/Ostm1 Cl}^-/\text{H}^+$  exchanger [137,140]. Interestingly, the disruption in mice of the  $\text{Ca}^{++}$ -activated  $\text{Cl}^-$  channel TMEM16A, which is highly expressed in several epithelia including those in lung, led to severe and eventually lethal defects in tracheal cartilage [231]. However, as demonstrated by other gene families (e.g.  $\text{ClC}$  proteins, or CFTR/ABC

transporters), one cannot take for granted that all members of a gene family perform similar transport functions. Hence, a classification of gnathodiaphyseal dysplasia as chloride channelopathy must await the biophysical demonstration that TMEM16E actually mediates anion currents.

Some members of the TMEM16 family are overexpressed in cancer cells. This includes TMEM16A [232,233] and TMEM16G (also known as NGEF), the latter being specifically expressed in the prostate [234] where it may affect cell–cell interactions [235]. It remains, however, unclear whether TMEM16G functions as a  $\text{Cl}^-$  channel and whether an upregulation of some TMEM16 proteins is promoting cancerous growth. It is interesting to note that also several different potassium channels [236] and transporters [237] were found to be overexpressed in cancer.

## 5. Ligand-gated chloride channels: GABA<sub>A</sub> and glycine receptors

GABA<sub>A</sub>-receptors and glycine-receptors are ligand-gated, pentameric anion channels that are opened by the respective neurotransmitter. In the adult nervous system, the associated chloride current is mostly inhibitory. Accordingly, mutations in GABA- or glycine-receptor subunits were found in human channelopathies that are associated with various symptoms of neuronal hyperexcitability.

### 5.1. GABA-receptors and epilepsy

Epilepsy, considered one of the most common neurological disorders (with a prevalence of 3 to 5 cases per 1000 persons, depending on age and ethnic background) is a heterogeneous group of syndromes which share episodic recurrent seizures as a common, central symptom. The different epilepsy syndromes are classified according to seizure type, typical age of onset, EEG findings and prognosis. Either genetic, environmental and/or developmental conditions can lead or predispose to seizures. Transient brain insults (such as ischaemia, neoplasia or infection) and many genetic disorders that influence neuronal metabolism or neuronal circuit assembly may lead to epilepsy. In these cases, epilepsy appears as part of a more complex phenotype and is classified as symptomatic epilepsy. Idiopathic epilepsies evolve in the absence of any apparent or suspected anomaly. Most idiopathic epilepsies show complex inheritance and may involve external factors (for a review, see [238,239]). Only few patients are affected by monogenic epilepsy syndromes that display a Mendelian pattern of inheritance. Many of these are due to mutations in either voltage-gated or ligand-gated ion channels. Thus, for instance, gain of function mutations in the  $\text{Na}^+$  channel genes *SCN1A* and *SCN1B* as well as in the  $\text{Ca}^{++}$  channel genes *CACNA1A*, *CACNA1H* and *CACNB4* have been associated to different types of idiopathic epilepsy syndromes. Loss of function mutations in  $\text{K}^+$  channel genes *KCNQ2* and *KCNQ3* or *KCNA1* have been associated with Benign Familial Neonatal Convulsions (BFNC) or with Focal Familial Seizures, respectively (for a review, see [240,241]). The purported role in epilepsy of mutations in the  $\text{Cl}^-$  channel gene *CLCN2* has been discussed above. Within the ligand-gated channel superfamily, mutations were found both in cation and anion channels. Mutations in the *CHRNA4* and *CHRN2* genes that encode the  $\alpha 4$  and the  $\beta 2$  subunits of the nicotinic acetylcholine receptor, respectively, were identified in Autosomal Dominant Nocturnal Front Lobe Epilepsy (ADNFLE). Notably, *GABRG2* and *GABRA1* genes, coding for  $\gamma 2$  and  $\alpha 1$  subunits of the GABA-receptor are associated to different monogenic epilepsy syndromes, whereas *GABRD* is considered a susceptibility gene. The role of GABA receptor in epilepsy syndromes is the focus of the present section.

GABA<sub>A</sub> receptors are ligand-gated chloride channels that are expressed in many areas of the CNS. They are targets of a range of sedative, hypnotic, anxiolytic, anti-convulsant and general-anaesthetic agents. In the adult mammalian brain, GABA<sub>A</sub> receptors are the major inhibitory neurotransmitter receptors, whereas they may be

excitatory in the embryonic and perinatal brain. The developmental change in the response to GABA (the 'GABA-switch') is the consequence of developmental changes in the intraneuronal concentration of  $\text{Cl}^-$ . Whereas  $[\text{Cl}^-]_i$  is above equilibrium in early development, it later decreases to well below its electrochemical equilibrium. This is owed mainly to an increased expression of the chloride extruder KCC2 [55,56,242]. Therefore, the opening of the anion channel pore by GABA leads to a depolarization in early development, but to hyperpolarization in most adult neurons. The postsynaptic inhibition of target neurons by GABA receptors is largely achieved by hyperpolarizing the postsynaptic membrane or by stabilizing the membrane potential close to its resting level (shunting inhibition). These receptors thus suppress both the spatial and temporal summation of excitatory postsynaptic potentials. Binding of the endogenous ligand GABA promotes the opening of the channel pore, allowing the flow of  $\text{Cl}^-$  and  $\text{HCO}_3^-$  down their electrochemical gradients.

Whereas ionotropic GABA<sub>A</sub> receptors are anion channels, metabotropic GABA<sub>B</sub> receptors regulate  $\text{K}^+$  and  $\text{Ca}^{++}$  channels through intracellular second-messenger pathways involving G-proteins. Like acetylcholine receptors, GABA<sub>A</sub> receptors are members of the superfamily of ligand gated channels. They are assembled from five subunits that surround a central ion-conductive pore. Each subunit has a large extracellular amino-terminal domain with a characteristic loop formed between two cysteine residues, a shorter carboxy-terminal domain and a membrane domain with four transmembrane segments (TM1 to TM4). Extensive evidence indicates that TM2 is lining the pore. GABA<sub>A</sub> receptors are allosterically modulated by barbiturates and benzodiazepines (BZ) in contrast to ionotropic GABA<sub>C</sub> receptors (reported in retinal horizontal cells) that differ in their kinetics and pharmacological properties. Specifically, BZ binds GABA<sub>A</sub> receptors at the junction between  $\alpha$  and  $\gamma$  subunits.

GABA<sub>A</sub> receptors are composed by subunits that are encoded by members of seven subfamilies (six  $\alpha$  subunits:  $\alpha 1$  to  $\alpha 6$ , three  $\beta$  subunits:  $\beta 1$  to  $\beta 3$ , three  $\gamma$  subunits:  $\gamma 1$  to  $\gamma 3$ , and one  $\delta$ ,  $\epsilon$ ,  $\pi$  and  $\theta$  subunits). Their most common stoichiometry is  $2\alpha:2\beta:1\gamma$ . The subunit composition differs among brain regions and cell types, thereby providing different modes of inhibition: i) a fast phasic activation of postsynaptic receptors to shape neuronal integration in the tens of ms scale [243], or ii) a tonic activation to modulate network excitability [244,245].

Mutations in both *GABRG2* gene, encoding the GABA  $\gamma 2$  subunit, and *GABRA1* gene, encoding the GABA  $\alpha 1$  subunit, have been linked to different forms of epilepsy. Initially, two missense mutations in the *GABRG2* gene were identified that co-segregated in a clear Mendelian inheritance pattern with a familial epilepsy syndrome, the so called Genetic Epilepsy with Febrile Seizure Plus (GEFS+) and Childhood Absence Epilepsy (CAE) [246]. Missense mutations in *GABRG2* are generally associated with the milder GEFS+ syndrome. A disease-associated K289M mutation in the first extracellular loop between the M2 and M3 transmembrane segments of the  $\gamma 2$  subunit decreased current amplitudes when co-expressed with  $\alpha 1$  plus  $\beta 2$  subunits in amphibian oocytes [246]. When co-expressed in HEK293 cells, an accelerated deactivation after GABA applications was found [247]. Both results indicate a loss of function.

Another missense mutation in *GABRG2*, R43Q, was found in affected members of a family with childhood absence epilepsy and Febrile Seizures (FS), a milder form of GEFS+ [248]. R43 is located on the first extracellular  $\text{NH}_2$ -terminal domain of the  $\gamma 2$  subunit. Whereas this mutation was initially described to abolish the sensitivity to BZ [248,249], it was later found to accelerate deactivation [249] or to decrease current amplitudes due to a reduced surface receptor [247,250,251]. Recent evidence suggests that mutant R43Q  $\gamma 2$  subunits are retained intracellularly upon heterologous expression, whereas  $\alpha\beta$  complexes are still being targeted to the plasma membrane [252]. In a knock-in mouse model for the R43Q mutation, which develops

absence seizures, the surface expression of the  $\gamma 2$  subunit was reduced, whereas the expression of the  $\alpha 1$  subunit remained unaltered. Hence a different subunit composition of surface-expressed receptor channels may underlie the altered receptor kinetics and thus neuronal excitability [253].

A different missense mutation, R139G, in the BZ coupling domain of the  $\gamma 2$  subunit, co-segregated with the disease in a Febrile Seizures (FS) family. In heterologous expression systems, receptor channels carrying R139G mutated  $\gamma 2$  subunits desensitized more rapidly than wild type receptors [254]. GEFS+ is characterized by a clinical phenotypic spectrum ranging from the milder symptoms observed in FS or FS+ (FS with offset beyond 6 years) syndromes to the more severe symptoms in the Severe Myoclonic Epilepsy of Infancy (SMEI). Truncation or nonsense mutations of the GABA receptor  $\gamma 2$  subunit are normally, but not always, linked to SMEI, whereas missense mutations are found in FS or FS+. As an exception to that rule, a splice site mutation at the 5'-end of intron 6 that is predicted to truncate the  $\gamma 2$  subunit before TM1 led to the mild FS [255]. However, no further data is available about this mutation. Furthermore, phenotypical variability may occur with the same mutation in the same family. For instance, a mutation (Q351X) on the  $\gamma 2$  subunit, in the intracellular loop between TM3 and TM4, was found in a family in which most members carrying the mutation displayed GEFS+, whereas a single individual presented with SMEI [256]. Taken together, these mutations cause neuronal hyperexcitability and hence epilepsy by reducing GABAergic inhibition through several different mechanisms such as disturbed assembly or trafficking or altered channel kinetics.

A missense mutation in *GABRA1*, the gene encoding the  $\alpha 1$  subunit, was linked to Autosomal Dominant Juvenile Myoclonic Epilepsy (ADJME) in a French Canadian family [257]. This mutation (A322D) was reported to reduce both current density and GABA sensitivity, findings that may be related to reduced surface expression and increased ER-degradation of the mutated subunit [258]. By contrast, S328X, a *de novo* mutation found in affected members of a family with sporadic Childhood Absence Epilepsy (CAE), produced truncated  $\alpha 1$  subunits that resulted in non-functional heteromeric GABA<sub>A</sub> receptors in the membrane surface [259].

A consistent correlation of mutations in different GABA-receptor isoforms with specific clinical types of epilepsy is not possible, in part owed to the rarity of these genetic syndromes. All mutations described so far are predicted to decrease GABAergic inhibition. However, results from heterologous expression studies often remain ambiguous. No clear correlation between clinical severity and alteration of electrophysiological properties of GABA receptors has been obtained to date. Another difficulty is the high variability of syndromes even between members of a given family, indicating important influences of other genes or environmental factors.

In addition to the role of GABA  $\gamma 2$  and  $\alpha 1$  subunit mutations in monogenic forms of epilepsy, genes encoding other GABA<sub>A</sub> receptor subunits have been suggested as epilepsy susceptibility genes. A case in point is *GABRD*, the gene encoding the GABA receptor  $\delta$  subunit. In a  $\alpha \beta \delta$  complex, it yields slow desensitizing receptors that are thought to mediate tonic inhibition. A missense mutation in its extracellular NH<sub>2</sub>-terminal domain, E177A, was found to contribute to GEFS+ by reducing maximal current at saturating GABA concentrations [260]. Surprisingly, a missense mutation (R220C) found in a GEFS+ family did not alter the current amplitude of recombinant receptors, whereas a different mutation in the same residue (R220H) decreased the peak amplitude of current. The latter mutation (R220H) was found both in patients (of IGE, GEFS+ and FS) and in control groups, thus possibly suggesting that this amino-acid exchange predisposes to polygenic epilepsies or be a neutral polymorphism. Therefore, larger cohorts are needed to confirm the significance of these R220 mutations in idiopathic epilepsy [261]. The  $\beta 3$  subunit is another example of a GABA receptor subunit with an ambiguous role in idiopathic epilepsy. Distinct haplotypes at the *GABRB3* promoter were linked to CAE in an

association study [262], endorsing the hypothesis of reduced *GABRB3* expression contributing to CAE. However, using a larger cohort this association could not be replicated [263].

Interestingly, the  $\beta 3$  subunit of the GABA<sub>A</sub> receptor plays a poorly understood role in craniofacial development since *GABRB3* disruption causes cleft palate in mice [264,265]. Furthermore,  $\beta 3$ -deficient mice display occasional epileptic seizures, consistent with impaired inhibition in the CNS. *GABRB3* has been suggested as a candidate of Angelman syndrome (AS) [266,267], a severe neurodevelopmental disorder accompanied by seizures. *GABRB3* is disrupted together with other genes within a large chromosomal deletion on 15q11–q13 in ~70% of AS cases. Furthermore, seizures in  $\beta 3$  subunit-KO mice showed a pharmacological response profile to antiepileptic medications similar to that observed in AS [268], suggesting a relative contribution of the *GABRB3* gene alone or in combination with other genes.

## 5.2. Glycine receptors and hyperekplexia

Glycine receptors (GlyR) also belong to the superfamily of pentameric, ligand gated ion channels. Like GABA<sub>A</sub> receptors, they mediate inhibitory synaptic transmission in the adult CNS. GlyRs are most abundant in interneurons of the brainstem and spinal cord, where they control motor rhythm generation, coordinate spinal reflex responses and modulate processing by sensory neurons. GlyRs are composed of  $\alpha$  and  $\beta$  subunits. There are four different  $\alpha$  subunits ( $\alpha 1$  to  $\alpha 4$ ) that display specific developmental and regional expression patterns and a single  $\beta$  subunit. The  $\alpha 1$  subunit is abundantly expressed in adult brainstem and spinal cord, partially overlapping with  $\alpha 3$ . In contrast,  $\alpha 2$  is mainly expressed in embryonic and neonatal neurons, with only minor levels being found in adult hippocampus and cortex. In rodent neonates, glycine receptors are  $\alpha 2$  homopentamers [269], whereas adult receptors are heteropentamers with a  $2\alpha:3\beta$  stoichiometry. Alternative splicing of  $\alpha$  subunits as well as post-transcriptional editing of a single amino acid of the  $\alpha 3$  subunit provide further heterogeneity of GlyRs (for a review see [270]). As discussed above, both GABA and glycine are inhibitory neurotransmitters in adults, but may be excitatory in embryonic and neonatal age, due to a positive Cl<sup>-</sup> equilibrium potential that causes Cl<sup>-</sup> efflux upon receptor activation.

Mutation of the *GLRA1* and *GLRB* genes cause hereditary hyperekplexia (HPX), a neurological disorder characterized by an exaggerated startle reflex upon mild auditory, tactile or visual stimuli. The startle reaction is followed by a period of generalized hypertonia in which voluntary movements are impossible. Since symptoms are manifest early after birth, HPX is also known as 'stiff baby syndrome'. It can lead to sudden death by laryngospasm or respiratory failure. HPX can often be easily diagnosed by a violent head retraction elicited by gently touching the patient's nose. Electromyographic studies in these patients reveal overexcitability and diminished inhibition [271]. Consciousness remains intact and intellect is usually normal although mild mental retardation may occur. In numerous families, HPX segregates as autosomal dominant with almost complete penetrance. Mutations in *GLRA1*, encoding for the  $\alpha 1$  subunit, account for about 80% of HPX. Several missense mutations have been identified. These are most often found in either the pore lining TM2, the intracellular loop between TM1 and TM2 or in the extracellular loop between TM2 and TM3. The most common mutations reported are R271L or R271Q [272]. They reduce glycine sensitivity and single-channel conductance [273,274] in recombinant expression systems. Similar effects have been observed in other HPX mutations such as Y279C, K276E, Q266H, and P250T.

Nonsense and missense mutations of the GlyR  $\alpha 1$  subunit have been associated to either autosomal dominant and recessive forms of HPX. Autosomal recessive forms of HPX have been described in sporadic cases, caused by homozygous nonsense mutations in *GLRA1*



(Y202X, R344X and S296X). Truncated subunits are unlikely to retain the ability to oligomerize with wildtype subunits and thus result in smaller glycine-gated currents without affecting the sensitivity to the agonist in heterologous expression systems. Autosomal dominant forms are often associated to missense mutations that alter channel gating, trafficking, and stability of GlyRs [275].

Only in one single case human mutations in *GLRB* have been described [276]. The patient who displayed transient HPX was a compound heterozygote for a missense (G229D) and a truncating mutation in *GLRB*. In heterologous expression,  $\alpha 1\beta$  (G229D) showed reduced sensitivity to agonists, suggesting a contribution of the  $\beta$ -subunit to agonist binding. No mutations in the other  $\alpha$  subunits have been found in humans.

Three spontaneous mouse mutants (i.e. *spastic* (*spa*), *spasmodic* (*spd*), and *oscillator* (*ot*)) display recessive hyperekplexia with symptoms such as exaggerated startle response and muscle rigidity [277–279]. These mice carry mutations in the GlyR  $\alpha 1$  subunit that result in either reduced GlyR levels [279–281] or reduced agonist sensitivity [282,283]. A transgenic mouse with symptoms more closely resembling the human disease was generated by introducing the R271Q mutation found in patients. These mice exhibit exaggerated startle response and inducible tremor that is correlated with a strong reduction of glycinergic inhibitory postsynaptic currents [280].

Not only mutations in genes for GlyR subunits, but also for other proteins involved in glycinergic inhibition can underlie HPX syndromes. For instance, mutations in gephyrin, a cytoplasmic protein that binds to GlyRs and which is important for their localization and clustering, or in the glycine transporter subtype 2 lead to HPX in humans and mice, respectively [284–286].

## 6. Conclusions and outlook

Chloride channelopathies are associated with astonishingly diverse phenotypes, highlighting the importance of anion transport both at the cellular and systemic level. We assume that more anion channelopathies remain to be discovered. Some knock-out mouse models have given phenotypes for which no corresponding human disease has been identified as yet, possibly because the corresponding human phenotypes are genetically heterogeneous and the ‘correct’ patient has not yet been analyzed for the corresponding gene. As demonstrated e.g. by skeletal abnormalities owed to mutations in *CLCN7*, also genetic diseases with no immediately intuitive connection to ion channel function may eventually turn out to be channelopathies. In view of the astonishingly large numbers of unrelated gene families encoding chloride channels, and considering that several anion channels identified electrophysiologically could not yet been assigned to particular genes, there may still be other families of chloride channels and associated diseases to be discovered. Significant progress in channelopathy research is expected from the fast advances being made in high-throughput sequencing techniques. This may soon allow massive genomic sequencing of genomic DNA from patients and control cohorts.

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